Effect of the nitrogen source on phycobiliprotein synthesis and cell reserves in a chromatically adapting filamentous cyanobacterium

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Cyanobacteria can utilize nitrate or ammonium as a source of fixed nitrogen for cell growth. In the filamentous *Calothrix* sp. strain PCC 7601, these two sources of nitrogen differently influenced the phycobiliprotein composition of the phycobilisomes, the major light-harvesting antennae. When compared to nitrate, growth in the presence of ammonium resulted in intracellular steady-state levels 35% lower for phycoerythrin and 46% higher for phycocyanin. Besides these differences in cell pigmentation, a rapid but transient accumulation of cyanophycin granule polypeptide occurred in ammonium-grown cells, while these macromolecules were not detected in cells grown with nitrate. In contrast, glycogen reserves displayed a dynamic pattern of accumulation and disappearance during cell growth which varied only slightly with the nitrogen source. The observed changes in cell pigmentation are reminiscent of the phenomenon of complementary chromatic adaptation, in which green and red wavelengths promote the syntheses of phycoerythrin and phycocyanin-2, respectively. As in complementary chromatic adaptation, the regulation of synthesis of phycoerythrin and phycocyanin-2 by the nitrogen source occurred mainly at the mRNA level. Moreover, the transcriptional start sites for the expression of the *cpeBA* and the *cpc2* operons, which respectively encode the two subunits of phycoerythrin and phycocyanin-2, were the same in cells grown in nitrate or ammonium, and identical to those in green- and red-light-grown cells. The results of this study suggest that acclimation to the spectral light quality and to the nitrogen source share some common regulatory elements.

Keywords: *Calothrix* sp. PCC 7601, light-harvesting antennae, nitrogen assimilation, cyanophycin, glycogen

INTRODUCTION

Cyanobacteria constitute a large and very diverse group of prokaryotic organisms performing oxygenic photosynthesis. Their photosynthetic apparatus is similar to that of plants except that light is harvested mainly by phycobiliproteins. These chromoproteins are organized into stable multimolecular structures, called phycobilisomes,

that are attached to the stromal surface of the thylakoid membranes. Phycobilisomes are composed of two domains: the central core proximal to the photosynthetic membrane, and six rods which radiate from the core. In *Calothrix* PCC 7601 the phycobilisome contains the blue-coloured phycobiliproteins allophycocyanin (AP) and phycocyanin (PC), and the pink-coloured phycoerythrin (PE). AP is located in the core substructure of the phycobilisome, while PC and PE form the peripheral rods (Bryant, 1987, 1991; Glazer, 1989; Grossman *et al.*, 1993).

The cyanobacteria have developed unique physiological and morphological features to respond to changes in environmental parameters, such as light and nutrients,

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Abbreviations: AP, allophycocyanin; PC, phycocyanin; PE, phycoerythrin; KU, Klett units; PPFD, photosynthetic photon flux density.

which allow them collectively to colonize nearly all ecosystems (Tandeau de Marsac & Houmard, 1993). For example, cells of *Calothrix* PCC 7601 can adjust their phycobiliprotein content to the wavelengths of the incident light available during growth. This phenomenon, called complementary chromatic adaptation, is characterized by a preferential synthesis of light-harvesting pigments with absorption spectra complementary to the incident light wavelengths, PE under green light and phycocyanin-2 (PC-2) under red light. In contrast, AP and phycocyanin-1 (PC-1) are synthesized independently of the spectral light quality (Tandeau de Marsac & Houmard, 1993). The interactions between the regulation of photosynthesis and nitrogen metabolism have received limited investigation in cyanobacteria. Nitrate reduction is known, however, to be a genuine photosynthetic process, which directly uses photosynthetically generated reductant (Guerrero & Lara, 1987).

Cyanobacteria can generally use nitrate, nitrite and ammonium as sole nitrogen sources for growth (Guerrero & Lara, 1987; Flores & Herrero, 1994). Some strains may reduce molecular nitrogen and grow at the expense of the ammonium that results from this process (Fay, 1992). When externally supplied, ammonium ions (NH_4^+) enter the cells via an active transport system, while the unprotonated form (NH_3) enters by diffusion and is trapped by protonation. Nitrate is taken up by cyanobacterial cells via an active transport system and is sequentially reduced to nitrite by nitrate reductase and then to ammonium by nitrite reductase. The glutamine synthetase (GS)–glutamate synthase (GOGAT) enzyme system is quantitatively the most important pathway for the assimilation of ammonium, either exogenously supplied or internally generated. As all nitrogen sources converge to the production of ammonium and glutamine, any physiological differences related to nitrogen sources must be due to the level of ammonium within the cell or to a direct effect of the nitrogen source on the regulation of cell metabolism.

Cyanobacteria accumulate different types of reserves that can be used as a source of either nitrogen, carbon, or both (Allen, 1984). Cyanophycin is a nitrogen reserve unique to, but not universally present in, cyanobacteria (Simon, 1973a, b; Allen & Hutchison, 1980; Lawry & Simon, 1982). These non-ribosomally synthesized polypeptides consist of equimolar quantities of arginine and aspartic acid that further assemble into granules of molecular mass ranging from 25 000 to 100 000 kDa (Simon, 1971, 1973a, 1976). Cyanophycin usually accumulates in cells growing with a nitrogen supply in excess of other essential nutrients, such as phosphorus and sulphur, but decreases under nitrogen-deficient growth conditions, where it serves as a nitrogen source (Allen *et al.*, 1980; Simon, 1973b). According to Carr (1988), cyanophycin granule polypeptides are a more dynamic nitrogen reserve than phycobiliproteins.

Glycogen is the major carbon and energy reserve compound accumulated by cyanobacteria during photoautotrophic growth (Smith, 1982). Accumulation of this

glucose polymer can occur as a result of nitrogen-limited growth conditions in the light or in the presence of an excess of utilizable carbon source (Lehmann & Wöber, 1976; Allen & Smith, 1969). Growth under suboptimal temperature conditions (van Eykelenburg, 1980), or a sudden increase in light energy input without changes in nitrogen metabolism, may also lead to glycogen accumulation (Ernst & Böger, 1985). In the dark or in the light, once conditions for balanced growth are re-established, glycogen reserves are rapidly degraded to yield energy and carbon for cell metabolism. Glycogen may therefore act as a reserve with dual functions of storage product and buffer substance between carbon fixation and carbon consumption in other biosynthetic pathways (Carr, 1988).

Phycobiliprotein synthesis depends on the supply of assimilable nitrogen in the environment, and these proteins may serve as a nitrogen reserve (Tandeau de Marsac & Houmard, 1993). Cyanobacteria incapable of fixing molecular nitrogen respond to nitrogen deprivation by degrading the phycobiliproteins and linker polypeptides that form the phycobilisomes, leading to a rapid cell bleaching (Allen, 1984; Bryant, 1987, 1991; Collier & Grossman, 1992, 1994). As originally postulated by Allen & Smith (1969) for *Anacystis nidulans* (*Synechococcus* sp. PCC 6301), phycocyanin acts as a nitrogen storage compound in *Spirulina platensis* (Boussiba & Richmond, 1980). In the marine cyanobacterium *Synechococcus* sp. strain DC2, free phycoerythrin is also a pool of stored nitrogen (Wyman *et al.*, 1985), of importance in marine environments, where nitrogen is frequently limiting.

The filamentous cyanobacterium *Calothrix* PCC 7601 has lost the capacity to differentiate functional heterocysts and to fix dinitrogen (Kallas *et al.*, 1985); combined nitrogen must therefore be provided for cell growth. To study the regulatory mechanism linking nitrogen assimilation and photosynthesis, we have examined the effect of the nature of the nitrogen source (nitrate or ammonium) on the synthesis of the phycobiliproteins, and on the accumulation of cellular nitrogen and carbon reserves in this chromatically adapting cyanobacterium.

METHODS

Organism and growth conditions. The filamentous cyanobacterium *Calothrix* sp. strain PCC 7601 (= *Fremyella diplosiphon* UTEX 481; hereafter designated *Calothrix* PCC 7601) was grown at 27 °C in Erlenmeyer flasks (1 l) containing 500 ml liquid BG-11 medium (Rippka *et al.*, 1979) buffered with filter-sterilized 10 mM NaHCO_3 and 10 mM HEPES/NaOH, pH 8.0. When ammonium was used as a nitrogen source, NaNO_3 was replaced by 5 mM NH_4Cl . Cultures were agitated with magnetic stirring bars and continuously gassed with air/ CO_2 (99:1, v/v). White light was supplied by fluorescent lamps (OSRAM L18W/25 Universal White) providing a photosynthetic photon flux density (PPFD) of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured with a LI-COR LI-185B quantum/radiometer/photometer equipped with a LI-190SB quantum sensor. Growth was followed by measuring Klett units (KU) with a Klett Summerson colorimeter, model 800-3, equipped with a red filter no. 66 transmitting light between 640 and 700 nm.

For the different experiments, a **stock culture** of nitrate-grown cells that had reached 200 KU was diluted to an initial cell density of 15 KU in fresh culture medium. These **precultures** contained either 17.6 mM NaNO₃ or 5 mM NH₄Cl as a nitrogen source. After 26 h, these actively growing precultures, which had reached approximately 100 KU, corresponding to an OD₇₅₀ of 0.5–0.6, were diluted 1:6 in fresh medium of identical composition, generating the **experimental cultures** from which samples were collected at different times during cell growth. For all the parameters presented in this paper, values given at time 0 correspond to those determined on samples of the precultures collected at 26 h. Aliquots of the same cell suspensions were immediately used to inoculate the experimental cultures. The purity of the cultures was checked at each step as described by Rippka *et al.* (1979). As *Calothrix* PCC 7601 cells are rather sensitive to lysis when culture conditions are changed, the above experimental strategy was chosen as the least stressful treatment. It provided cells in a very reproducible physiological state to start the experimental cultures. Like some other cyanobacteria, *Calothrix* PCC 7601 cells do not take up nitrate when ammonium is available as a nitrogen source (Martín-Nieto *et al.*, 1989). The nitrate concentration of 0.08 mM (determined by using the kit from Boehringer Mannheim, ref. 90565) at the onset of the experimental cultures containing ammonium remained constant until the end of the experiments. Therefore any effect of nitrate on the results could be excluded.

Preparation of crude cell-free extracts. Cell samples from both culture conditions described above, corresponding to approximately 50 µg chlorophyll *a*, were harvested by centrifugation (15 min, 8000 *g*, 20 °C). The pellets were washed twice with 20 mM sodium acetate buffer, pH 5.5, containing 10 mM Na₂EDTA and 500 µM Pefabloc (Pentapharm AG), as protease inhibitors, and stored at –20 °C until analysis. Each pellet was resuspended to a final volume of 3.7 ml in the same buffer. The thawed cell suspension was then subjected to two passages through a mini French pressure cell at 135 MPa. Complete cell breakage was confirmed by microscopic examination of the crude extract.

Phycobiliprotein determination. Crude cell-free extract (1.9 ml) was precipitated with 100 µl of a 200 mg ml^{–1} streptomycin sulphate solution for 30 min at 4 °C and centrifuged at 18000 *g* for 10 min at 4 °C, in order to eliminate membrane fragments containing chlorophyll *a* (Tandeau de Marsac & Houmard, 1988). The supernatant was supplemented with 1 mM dithiothreitol. The amounts of PE, PC and AP were calculated from measurements of the absorbances at 565, 620 and 650 nm as previously described (Tandeau de Marsac & Houmard, 1988). These measurements do not distinguish between the constitutive PC-1 and the inducible PC-2, which differ only slightly spectroscopically (Bryant & Cohen-Bazire, 1981).

Chlorophyll *a* determination. Crude cell-free extract (0.5 ml) was extracted with 90 % (v/v) methanol for 1 h at 4 °C in dim light, followed by centrifugation at 10000 *g* for 15 min at 4 °C. The chlorophyll *a* content was calculated from the absorbance of the methanolic extract at 665 nm (Tandeau de Marsac & Houmard, 1988).

Protein determination. The protein content of crude cell-free extracts (1 ml) was determined by the Lowry method, with bovine serum albumin as standard, as described by Tandeau de Marsac & Houmard (1988). The Lowry method does not detect cyanophycin granule polypeptide, composed of polymers of arginine and aspartate.

Extraction and determination of cyanophycin granule polypeptide. Samples were prepared as described for crude cell-free extracts except that cell pellets were washed with sterile distilled H₂O. Cell samples for cyanophycin detection corresponded to approximately 50–80 µg chlorophyll *a* for ammonium-grown cultures. The lack of cyanophycin in nitrate-grown cultures was confirmed on larger volumes, corresponding to as much as 200 µg chlorophyll *a*. Cyanophycin granules were isolated from crude cell-free extracts and their arginine content was measured by the modified Sakaguchi reaction as described by Simon (1973b). The arginine content was then converted to cyanophycin granule polypeptide as described by Allen & Weathers (1980). Values represent the means of two determinations on duplicate samples treated independently.

Glycogen determination. Cell suspensions, corresponding to 7–15 µg chlorophyll *a*, were concentrated to 1 ml by centrifugation (15 min, 8000 *g*, 20 °C) and stored at –20 °C until analysis. Samples (200 µl) were hydrolysed by boiling for 20 min with 10 µl 50 % (v/v) H₂SO₄. One millilitre of *o*-toluidine reagent (Sigma, ref. 635-6) was then added. Samples were boiled for 10 min, cooled on ice, and the concentration of glucose released by the acid hydrolysis was calculated from the absorbance at 635 nm using a standard curve established with a glucose solution (Sigma, ref. 635-100). Values represent the means of two determinations made on samples from two or three independent cultures.

Dry weight determination. Cell suspensions, corresponding to approximately 70 µg chlorophyll *a*, were harvested by centrifugation (15 min, 27000 *g*, 20 °C). Cell pellets were washed twice and resuspended in 20 ml sterile distilled H₂O. Cell suspensions were then divided into three aliquots which were harvested by centrifugation, washed once and resuspended in a minimal volume of sterile distilled H₂O. These samples were dried in aluminium cups at 60 °C for at least 3 d, to permit complete liquid evaporation before weighing.

Ammonium determination. The concentration of ammonium in the culture medium was determined by adding 50 µl Nessler reagent (Sigma, ref. 14-2) to 1 ml of samples which were appropriately diluted with fresh BG-11₀ culture medium devoid of ammonium chloride (Rippka *et al.*, 1979). The absorbance at 395 nm was immediately determined and ammonium concentration was calculated by reference to a standard curve of 30–200 µM ammonium chloride in medium BG-11₀. Values represent the means of three to six determinations made on samples from two independent cultures.

RNA preparation, hybridization with ³²P-labelled probes, and primer extension. Cell suspensions corresponding to approximately 200 µg chlorophyll *a* were harvested by filtration through Millipore filters (8 µm pore size). Cells were resuspended in 650 µl cold buffer (10 mM sodium acetate pH 4.5, 200 mM sucrose, 55 mM Na₂EDTA). After addition of 1 g sterile glass beads (0.11 mm diameter, Braun), the cell samples were frozen in liquid nitrogen and kept at –20 °C until analysis. Total RNA was prepared by the procedure described by Mazel *et al.* (1986) with the following modifications: 0.3 ml of 3 % (w/v) Bentone MA (previously called Macaloid; Rheox Company, NL Industries, PO Box 700 Highstown, NJ 08520, USA) in 0.1 M Tris/HCl, pH 8.0, 70 µl of 20 % (w/v) sodium dodecyl sulphate and 1.5 ml of phenol [saturated with 100 mM Tris/HCl, 1 mM Na₂EDTA, pH 8.0, and containing 0.1 % (w/v) 8-hydroxyquinoline] were added to the frozen samples. Cell disruption was achieved by eight pulses of 30 s of vortexing at maximal speed, with an interval of 30 s between each pulse to prevent heating. Cell extracts were then centrifuged at 8000 *g* for 15 min

at 4 °C. The aqueous phases were collected, adjusted to 1 M with NaCl, and total RNA precipitated with 2 vols cold ethanol for 2 h at -20 °C. After centrifugation at 12000 *g* for 30 min at 4 °C, each pellet was resuspended in 100 µl H₂O and RNA was again precipitated with cold ethanol in the presence of 0.3 M sodium acetate pH 5.2. Each RNA sample, containing approximately 100–150 µg total RNA, as determined by absorbance at 260 nm, was resuspended in H₂O to a final concentration of 2 µg µl⁻¹. Aliquots were kept at -70 °C until use. Sterile distilled H₂O treated with DEPC (diethylpyrocarbonate) was used throughout the procedure described above.

Samples containing 12 µg total RNA per lane were electrophoresed for 16–18 h at 1.6 V cm⁻¹ through 1.6% (w/v) agarose gels. Both the gel and the running buffers contained 0.05 M HEPES/NaOH, 1 mM Na₂EDTA, pH 7.8, and 16% (v/v) formaldehyde. Blotting and hybridization procedures were as described by Campbell *et al.*, (1993). The DNA probes were as follows: AP, a 1.1 kb *Dra*I fragment encoding AP subunits α and β (*apcA1B1*) (Houmard *et al.*, 1988); PC-2, a 0.4 kb *Ssp*I fragment located just upstream from the ATG codon and carrying the transcribed but untranslated region of the *cpc2* operon (*cpcB2A2H2I2D2*) (Tandeau de Marsac *et al.*, 1988); PE, a 1.1 kb *Xba*I-*Eco*RI fragment encoding part of the β and α PE subunits (*cpeB* and *cpeA*) (Mazel *et al.*, 1986); PC-1, a 0.36 kb *Hind*III fragment of pPM114 carrying part of the *cpc1* promoter and the beginning of the β subunit of PC-1 (Schyns *et al.*, 1994). To quantify the relative transcript levels, the radioactivity of the hybridizing bands on the RNA blots was determined by scanning photoactivable screens on a Molecular Dynamics 445SI PhosphorImager. All quantifications, data display and analysis were performed using Molecular Dynamics Image Quant software. A 120 bp *Xcm*I-*Pvu*II restriction fragment internal to the *Calothrix* PCC 7601 *rnpB* gene encoding the RNA subunit of ribonuclease P (Vioque, 1992) was used as a probe to quantify the amount of RNA loaded and transferred to the filters, and to standardize the measurements. The steady-state levels of the *rnpB* transcripts from ammonium- and nitrate-grown cells correlated well with the abundance of total rRNAs as visualized on agarose gels after staining with ethidium bromide.

For primer extensions, 20 µg total RNA was precipitated with 10⁶ c.p.m. of radioactively end-labelled primers and the reactions were performed as described by Golden *et al.* (1986) except that annealings between RNAs and primers were performed at 37 °C for 16 h. AMV reverse transcriptase (Boehringer) was used in a PXII buffer from which KCl was omitted. The oligonucleotides used as primers were: 5'-GCATCTGCTGAACTACAGC-3', complementary to part of the nucleotide sequence downstream from the initiation codon of the *cpeB* gene (Fig. 5b), and 5'-GCCTCTAGTAT-CAGCCTGGG-3' and 5'-CAATTCCCTTGGCAATCAGG-3', complementary to parts of the nucleotide sequences located downstream and upstream, respectively, from the initiation codon of the *cpcB2* gene (Fig. 5b).

RESULTS

Growth in the presence of nitrate or ammonium

Several growth parameters were measured in order to identify quasi-steady-state conditions under which to compare the physiological states of growth arising from different nitrogen sources. Cyanobacteria are often susceptible to ammonium poisoning under suboptimal growth conditions (Abeliovitch & Azov, 1976; Boussiba & Gibson, 1991) and NaHCO₃ is necessary for am-

Fig. 1. Effect of the source of fixed nitrogen on growth of *Calothrix* PCC 7601 cells, measured as dry weight (+, nitrate-grown cells; Δ , ammonium-grown cells), total cell protein ($\diamond$, nitrate-grown cells; $\times$, ammonium-grown cells) and chlorophyll *a* (chl *a*) ($\circ$, nitrate-grown cells; $\square$, ammonium-grown cells). Error bars represent $\pm$ SD of three independent replicates. Values for dry weight and total cell protein are the means of three determinations on the same culture.

monium uptake (Boussiba *et al.*, 1984). Therefore, the culture medium was supplemented with NaHCO₃ and HEPES to maintain the pH between 7.5 and 7.8 during growth. Under these conditions, ammonium chloride at 5 mM permitted good growth of *Calothrix* PCC 7601. Preliminary experiments showed that oxygen evolution was similar in nitrate- and ammonium-grown cells when measured under a PPFD of 50 µmol m⁻² s⁻¹ supplied by an incandescent lamp, indicating that ammonium at that concentration did not uncouple linear electron transport (data not shown).

To perform the experiments described in this paper, a **stock culture** grown in nitrate was diluted and grown as a **preculture** in the nitrogen source of choice (nitrate or ammonium) for 26 h, followed by a second dilution to form the **experimental culture**, from which measurements were derived (see Methods for details). Doubling times, based on dry weight, total cell protein or chlorophyll *a* content, were 6–7 h for the cultures grown with either ammonium or nitrate as the source of fixed nitrogen (Fig. 1). Growth proceeded exponentially for at least 20 h after the dilution of the cultures. We thus chose for this study to measure all parameters during this period of stability and to express results on a chlorophyll *a* basis.

Influence of the nitrogen source on the phycobiliprotein content

Preliminary experiments showed that *Calothrix* PCC 7601 cells grown under white light and in the presence of nitrate were brown-red in colour. If such nitrogen-replete cells were transferred to a medium containing ammonium, they became greenish within less than two generations.

Fig. 2. Effect of the source of fixed nitrogen on the phycobiliprotein content of *Calothrix* PCC 7601 cells. (a, b, c) Phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (AP), respectively, from cultures grown in the presence of nitrate (—○—) or ammonium (---□---), expressed as μg phycobiliprotein (PB) per μg chlorophyll *a* (chl *a*). (d) Total PB from nitrate- (—●—) or ammonium-grown cells (---■---), expressed as μg PB per μg chl *a*, and (PE+PC)/AP ratios from nitrate- (—○—) or ammonium-grown cells (---□---). Error bars represent $\pm$ SD of three independent replicates.

After reaching stationary phase, cells recovered a slightly brown-reddish colour, but upon dilution to a fresh medium containing ammonium they again displayed a similar cycle of changes in pigmentation. When exponentially growing in a medium containing both nitrate and ammonium, cells exhibited the same greenish phenotype as with ammonium alone. Similarly, cells that had experienced 12 h of nitrogen starvation became green or brown-red after addition of ammonium or nitrate, respectively (data not shown).

In order to examine the synthesis of phycobiliproteins during steady-state growth on nitrate as compared to ammonium, cells were incubated in the presence of either nitrogen source, and the pigment content of the cells was determined on samples removed at different times (Fig. 2a–d). The AP content of cells grown in the presence of either nitrate or ammonium did not differ significantly, since the AP/chlorophyll *a* ratio remained approximately 1.5 under both growth conditions (Fig. 2c). In contrast, the nitrogen source markedly influenced the PE and PC content of the cells. PE was 35% lower in ammonium-compared to nitrate-grown cells (Fig. 2b), whereas PC was 46% higher (Fig. 2a). In ammonium-grown cells the PE/PC ratio was about 1, indicating that the phycobilisomes contain the same number of PE and PC discs, whereas this ratio was about twofold higher in nitrate-grown cells (compare Figs 2a and 2b). The ratios of total phycobiliproteins to chlorophyll *a*, and of PE+PC to AP, which are proportional to the number of phycobilisomes per unit photosynthetic membrane and the number of peripheral discs per phycobilisome, respectively, remained unchanged whatever the source of nitrogen (Fig. 2d).

These results indicate that the nitrogen source specifically modulates the composition of the phycobilisome rods, but neither their length nor the total number of phycobilisomes per unit photosynthetic membrane.

Accumulation of cyanophycin and glycogen reserves

No cyanophycin granules were visible by light microscopy in nitrate-grown cells of *Calothrix* PCC 7601, at any phase of growth examined. In contrast, a preculture incubated in an ammonium-containing medium exhibited, upon transfer to experimental culture conditions, a transient accumulation of these structured granules, which appeared dispersed throughout the cytoplasm. A rapid accumulation of cyanophycin granule polypeptide occurred over the first 5 h (Fig. 3a). Subsequently, the specific rate of synthesis decreased and finally ceased, resulting at 20 h in the same specific content of this polypeptide as that observed at 1 h (Fig. 3a). At 11 h after transfer, the amount of cyanophycin granule polypeptide corresponded to approximately 5% of the cell dry weight. The accumulation of cyanophycin granules occurred in parallel with an initial consumption of ammonium from the culture medium that was significantly higher than that observed during subsequent growth (Fig. 3a). After 26 h (corresponding to a culture density of 100 KU) of growth in the presence of ammonium, cells were in late exponential phase and no longer contained any cyanophycin granules, although the culture medium still contained 2 mM ammonium (data not shown).

The glycogen content of nitrate- and ammonium-grown cells was measured in the same time-course experiment (Fig. 3b). Upon dilution of the culture, cells exhibited a

Fig. 3. Time-course experiment showing the accumulation of cyanophycin (a) and glycogen reserves (b) in nitrate- and ammonium-grown cells of *Calothrix* PCC 7601, and consumption of ammonium in the culture medium (a). Cyanophycin and glycogen in nitrate- (—○—) or ammonium-grown cells (---□---) are expressed as $\mu\text{g per } \mu\text{g}$ chlorophyll *a* (chl *a*), and ammonium concentration remaining in the culture medium (---■---) in mM. After 26 h growth in the presence of ammonium, cells no longer contained any cyanophycin granules (data not shown).

rapid glycogen accumulation, at the same rate [approximately $1.2 \mu\text{g glycogen } (\mu\text{g chl } a)^{-1} \text{ h}^{-1}$] irrespective of the nitrogen source. The glycogen content of the cells was 2.5–3 times higher at 5 h than at 0 h and represented approximately 17% of the dry weight. After 5 h, the rate of glycogen accumulation decreased under both growth conditions but remained slightly higher in nitrate-grown cells. As the net increase in glycogen became smaller than that of chlorophyll *a*, the specific glycogen content of the cells decreased in both ammonium- and nitrate-grown cells and at 20 h resulted in levels similar to those observed at the onset of the experiment.

Thus, transient increases in cyanophycin granule polypeptides and glycogen occurred under the same standard conditions that led to stable phycobiliprotein levels.

Effect of nitrate and ammonium on phycobiliprotein transcripts

To investigate the molecular basis for the difference in phycobiliprotein synthesis during cell growth in the

presence of either ammonium or nitrate, we performed RNA/DNA hybridizations. Quantifications of transcript levels were made by scanning radioactivity of the RNA blots presented in Fig. 4(a) and normalizing the signals to the *rnpB* transcripts in each lane. During the first hour following the transfer of the cells to experimental culture conditions, the steady-state levels of the transcripts corresponding to the *apc1*, *cpc1*, *cpc2* and *cpeBA* operons decreased to different extents in ammonium-grown cells (Fig. 4b). In nitrate-grown cells, this decrease was less pronounced for the *cpc1* and *cpeBA* transcripts; in contrast, the *cpc2* and *apc1* transcripts remained about constant. After a rapid increase of all transcripts to reach a maximum level between 5 h and 11 h, their abundance subsequently declined to a level close to that observed at 0 h. These transient changes in the abundance of the mRNAs at different times during cell growth were neither accompanied by nor followed by significant change in the amounts of the corresponding gene products (Fig. 2a–c).

Fig. 4(c) expresses the relative abundance of each phycobiliprotein transcript as the ratio of that observed in ammonium- to that of nitrate-grown cells. There were slightly fewer *apc1* transcripts under the former conditions. The ratios of *cpc1* transcripts decreased during the first hour following the transfer to a fresh medium but then returned to about a mean value of 1.25, similar to the level at zero time. The ratios of the *cpc2* mRNAs were more variable, but the mean value was higher than those obtained for the *cpc1* transcripts. In contrast to *cpc2*, there were fewer *cpeBA* mRNAs in ammonium- than in nitrate-grown cells. These plots, together with those presented in Fig. 4(b), demonstrate the opposing effects of the two nitrogen sources on the abundance of the *cpc2* and *cpeBA* transcripts, and also reveal that the *cpc1* and *apc1* transcripts are less affected. The overall differences in mRNA abundances under the two nitrogen regimes are in good agreement with the spectroscopic data obtained for the corresponding gene products PE and PCs (PC-1 and PC-2) (Fig. 2a, b). In the case of *apc1*, the corresponding gene product AP was the same under both nitrogen conditions (Fig. 2c) despite the 20% difference in transcript abundance.

Primer extension experiments were performed with RNA isolated from nitrate- and ammonium-grown cells to test whether variations in the relative abundance of the *cpeBA* and the *cpc2* mRNAs reflected different uses of specific transcription start sites under the two regimes of fixed nitrogen. Results showed that transcription started at the same site for the *cpeBA* and the *cpc2* operons irrespective of the nitrogen source provided for cell growth (Fig. 5a). A primer complementary to nucleotides at position 144–163 upstream from the first initiation codon of the *cpcB2* gene generated a single extension product (Fig. 5a and P1 in Fig. 5b), while a primer located at nucleotides 48–29 revealed three additional minor products (P2, P3 and P4 in Fig. 5b). All these transcription start sites were identical to those obtained for the *cpeBA* and the *cpc2* operons with cells grown under green and red light, respectively (data not shown).

DISCUSSION

In the present work, we show that the nitrogen source specifically alters the phycobiliprotein content of *Calothrix* PCC 7601 cells. PE and PC levels vary in opposite directions, the former being lower in ammonium- than in nitrate-grown cells and the latter higher under the same conditions. The overall phycobilisome number and rod length remain, however, similar in nitrate- and ammonium-grown cells, with only the specific composition of the rods being modified. Considering that a PE monomer carries five tetrapyrrole chromophores and a PC monomer only three (Glazer, 1989), it can be estimated that at least 10% fewer chromophores are required to build up phycobilisomes in ammonium- than in nitrate-grown cells. As phycobiliproteins represent approximately 25% of the total cell protein under the present experimental conditions, this would correspond to a substantial economy in the use of glutamate molecules, the substrate of the first enzyme involved in the synthesis of tetrapyrrole chromophores in cyanobacteria (Beale, 1994). The benefits of such an economy are not readily evident. One could postulate, however, that the changes in phycobiliprotein and chromophore content are a means of adjusting the photosynthetic activity, in such a way that the production of reducing power does not exceed its demand, which is expected to be lower in ammonium- than in nitrate-grown cells.

Our results provide quantitative data confirming the work of Erokhina (1992), who observed, for a few cyanobacteria, changes in the absorption spectra of cells and phycobilisomes by varying the source of fixed nitrogen. In *Synechococcus* sp. strain PCC 7002, which is not a PE-containing cyanobacterium, de Lorimier *et al.* (1992) found that phycobilisomes isolated from ammonium-grown cultures consistently contained 10–20% more phycocyanin than those grown with nitrate. In *Calothrix* PCC 7601, we did not observe any significant difference in the total phycobiliprotein content between ammonium- and nitrate-grown cells, as there is a compensation due to a modulation of the syntheses of PC and PE in opposite directions. This modulation might concern PE-containing strains in general or be specific only for those able to undergo complementary chromatic adaptation.

Calothrix PCC 7601 cells transiently accumulate cyanophycin granule polypeptide when grown in the presence of ammonium. Accumulation and disappearance of this nitrogen reserve are correlated with the rapid decline of ammonium concentration in the culture medium during growth, but not with the effect of ammonium on phycobiliproteins, since cyanophycin reaches a maximum and then disappears without noticeable changes in the synthesis of these chromoproteins. Cyanophycin storage could not, however, be the unique means to avoid intracellular ammonium toxicity as cyanophycin represented, on a N atom basis, at most 5% of the ammonium consumed from the culture medium. When the ammonium uptake slows, the specific rate of cyanophycin granule polypeptide synthesis declines. These reserves are then mobilized, since after a decrease in the

net cell content from 11 h after transfer, their steady-state level returns to zero by 26 h. This accumulation and decrease of cyanophycin granule polypeptide may well be due to threshold effects of the external concentration of ammonium during growth. However, the experiments were initiated with a dilution of the cultures; therefore other factors might be involved, such as increased light irradiance, replenishment of a minor constituent of the medium or dilution of a substance that accumulated during growth.

Cyanobacteria generally accumulate cyanophycin granule polypeptide as a reserve compound during the transition from exponential to stationary phase (Simon, 1973b; Allen *et al.*, 1980). These polypeptides can also be synthesized immediately following the addition of a usable source of fixed nitrogen to nitrogen-starved cells (Rippka & Stanier, 1978; Allen & Hutchison, 1980) or, as in *Anabaena cylindrica*, following addition of ammonium to cells previously acclimated to N₂-fixing conditions (Mackerras *et al.*, 1990). As in *A. cylindrica*, the accumulation of cyanophycin granules in *Calothrix* PCC 7601 was rapid and transient, and occurred in nitrogen-replete cells, since the preculture still contained 2.0 mM ammonium at the time of transfer to fresh medium. Cells grown on nitrate did not, however, contain cyanophycin granule polypeptide at any phase of growth. The levels of nitrate and nitrite reductase activities (or syntheses) might thus be too low to produce ammonium in amounts sufficient to stimulate the enzymes involved in arginine and aspartate syntheses. In *A. cylindrica* cells, for example, the high concentration of glutamine resulting from the assimilation of exogenously supplied ammonium indeed favours arginine synthesis, via the carbamoyl phosphate–citrulline pathway (Ohmori & Ohmori, 1990). Furthermore, a glutamate–aspartate aminotransferase activity can drive the formation of aspartate after incorporation of NH₄⁺ into glutamine and glutamate (Flores & Herrero, 1994). Arginine and aspartate thus synthesized can then polymerize to form cyanophycin granule polypeptides. If such enzymic pathways prove to function similarly in *Calothrix* PCC 7601 cells, they could contribute to the high production of cyanophycin granule polypeptides specifically in ammonium- and not in nitrate-grown cells.

Mackerras *et al.* (1990) suggested that the carbon skeletons for cyanophycin synthesis may originate from the degradation of glycogen following addition of nitrogen to starved cells. In *Calothrix* PCC 7601 cells grown under the present conditions, however, accumulation of cyanophycin granule polypeptide occurred concomitantly with an accumulation of glycogen. Indeed, the net glycogen content of the cells reached a maximum shortly after inoculation into fresh culture media and then decreased, with only a slight difference between ammonium- and nitrate-grown cells. This initial rapid glycogen storage probably resulted from an enhancement of photosynthetic activity due to an increase of light irradiance upon dilution of the cultures. Sudden changes in irradiance may affect the carbon/nitrogen ratio and induce glycogen accumulation in cyanobacterial cells irrespective of the nitrogen

Fig. 4. (a) RNA blot analysis of the *apcA1B1*, *cpcB1A1*, *cpeBA*, *cpcB2A2* and *rnpB* transcripts from *Calothrix* PCC 7601 cells grown in nitrate or ammonium. The sizes (in kb) of the transcripts encoding α and β subunits of phycobiliproteins are: 1.4 for *apcA1B1*, 1.6 for *cpcB1A1* and *cpcB2A2*, and 1.5 for *cpeBA*. The minor larger mRNA species corresponding to the

Fig. 5. (a) Primer extension analysis of the *cpeBA* and *cpc2* mRNAs in *Calothrix* PCC 7601 cells grown in nitrate or in ammonium. The sequencing ladder (complementary to the sense strand) was generated by using the same primer as that employed for the extension reactions. Transcription start points are indicated on the left-hand side by arrows. (b) Nucleotide sequence (sense strand) of the promoter regions of the *cpeBA* and *cpc2* operons. Nucleotides are numbered with respect to the initiation codons. The different transcription start sites determined in different laboratories are indicated as follows. For the *cpeBA* operon: $\blacklozenge$, Federspiel & Grossman (1990); $*$, Tandeau de Marsac et al. (1988); arrows, this study. For the *cpc2* operon: Δ , Conley et al. (1988); $\diamond$, V. Capuano & J. Houmard, unpublished data and Sobczyk et al. (1994); arrows, this study. The oligonucleotides used as primers were complementary to the underlined nucleotide sequences.

source (Ernst et al., 1984; Ernst & Böger, 1985; De Philippis et al., 1992). In contrast, the subsequent decrease in the net cellular glycogen content might depend on the nitrogen source. In *Anacystis nidulans* (*Synechococcus* sp. PCC 6301), nitrogen assimilation can affect the rate of CO_2 fixation, decreasing incorporation of carbon into carbohydrates and enhancing carbon flow to organic and amino acids at the expense of the glycogen reserve. These effects are even more pronounced in the presence of ammonium than with nitrate (Romero & Lara, 1987; García-González

et al., 1992; Coronil et al., 1993). Such a control seems to occur in *Calothrix* PCC 7601 and predominates in the later phase of the exponential growth of cells in the presence of ammonium. The decrease of glycogen accumulation occurred over a time period when cyanophycin granule polypeptide synthesis ceased. These phenomena may be related, as an enhanced incorporation of carbon into compounds other than glycogen may occur in response to an increased intracellular nitrogen content corresponding to cyanophycin disappearance.

transcription of the entire *apc1* and *cpc2* operons are not presented. The prints of the autoradiograms corresponding to the hybridizations with the *apcA1B1* and *cpeBA* (left-hand side) and *cpcB1A1* and *cpcB2A2* (right-hand side) DNA probes were cut and assembled together for convenience. Middle panels: the same two RNA blots were hybridized with a DNA probe of the *Calothrix* PCC 7601 *rnpB* gene to provide an estimate of the RNA loading. The *rnpB* transcript is about 0.5 kb. (b) Time course of the variation in the transcript levels of the *apcA1*, *cpc1*, *cpeBA* and *cpc2* operons in nitrate- (—○—) or ammonium-grown cells (---□---). Values relative to the *rnpB* transcript level were obtained after scanning of the RNA blots and correspond to the sum of all the transcripts for each of the operons, of which only the major transcripts are presented in (a). (c) Changes in the transcript levels of the *apcA1*, *cpc1*, *cpeBA* and *cpc2* operons in response to the nitrogen source. The plots show the ratios of transcripts in ammonium-grown cells to those of nitrate-grown cells (values of transcript levels determined as in b).

The effect of ammonium on the synthesis of phycobiliproteins resembles to some degree the red-light effect observed during complementary chromatic adaptation. In *Calothrix* PCC 7601, the regulation of the expression of the *cpeBA* and *cpc2* operons under green and red light, respectively, operates mainly at the transcriptional level, and involves specific DNA-binding proteins that recognize the promoter regions (Tandeau de Marsac & Houmard, 1993; Sobczyk *et al.*, 1993, 1994). The present study shows that the regulation by the nitrogen source also operates at least in part at the mRNA level, with fewer *cpeBA* and more *cpc2* transcripts in ammonium- than in nitrate-grown cells. The transcript abundance of the *cpc1* genes was also somewhat higher in ammonium-grown cells. A similar effect has been described in complementary chromatic adaptation, with a slightly greater level of *cpc1* mRNAs in red- than in green-light-grown cells (Conley *et al.*, 1986). In the case of the *apc1* operon the difference in the transcript levels, which is not correlated with a change in the AP content between nitrate- and ammonium-grown cells, is more questionable as it represents approximately 20%, a value which could be within the experimental errors intrinsic to such quantifications. If this difference is significant, however, it would indicate a post-transcriptional regulation of the expression of the *apc1* operon.

Although a slow turnover of the phycobiliproteins cannot be excluded, a post-transcriptional regulation might also occur for the expression of the other phycobiliprotein operons, as the transient changes in transcript abundance observed during growth do not affect the level of the corresponding proteins. A stress effect resulting from cell dilution could explain the decrease in transcript abundance 1 h after transfer of the cells to a fresh medium, which was probably too brief to be reflected in phycobiliprotein cell content. In contrast to this stress effect seen with both nitrogen sources, there is a specific effect of ammonium on transcription and synthesis of phycobiliproteins, maintained over at least three cell generations – too long for a stress effect to persist.

The transcription start sites determined for the *cpeBA* and *cpc2* operons, in nitrate- or ammonium-grown cells, are the same as those identified for the *cpeBA* and *cpc2* operons in green- and red-light-grown cells, respectively. Fig. 5(b) shows a comparison of the transcription start sites determined in the present work with those already published. The transcription start sites identified for the *cpeBA* operon are very close to those previously found. The P1 transcription start site for the *cpc2* operon is located a few nucleotides from the transcription start sites found for the *cpc2* operon in red-light-grown cells. The size of the mRNA expected from this transcription start site corresponded to that of the transcript revealed by RNA/DNA hybridization. Three additional minor extension products (P2, P3 and P4) were also detected which were not identified by previous S1 mapping experiments and did not correspond to any stable full-length mRNAs after hybridization of the RNA blots with DNA probes from the *cpc2* operon. These additional products might in fact be due to the presence of mRNA secondary structures

to which the technique of primer extension is more sensitive than is S1 mapping.

Previous studies with strains of *Calothrix* sp. have shown that: (i) both the intensity and the spectral quality of the light affect phycobiliprotein synthesis; and (ii) both the spectral light quality and the nitrogen supply can control cell differentiation processes, such as those of heterocysts and hormogonia (Campbell *et al.*, 1993; Campbell, 1994). The similar specific effects of fixed nitrogen source and light spectral quality on phycobiliprotein synthesis presented in this report support the idea that there are common regulatory mechanisms, or at least cross-talk, between nitrogen assimilation and complementary chromatic adaptation to control phycobiliprotein gene expression and thus optimize the collection of light energy for balanced cell growth. Our results provide new clues to understanding these interactions between nitrogen assimilation, photosynthesis and cell differentiation in *Calothrix* PCC 7601.

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